Incorporation of Radioactive Amino Acids into Proteins of the Microsome Fraction of Guinea-Pig Liver in a Cell-Free System

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Although the synthesis of specific soluble proteins has rarely, if ever, been unequivocally demonstrated to occur in cell-free preparations of mammalian cells, the capacity for the uptake of amino acids into peptide linkage in 'non-specific' protein is less readily destroyed by damage to the cell. Thus the incorporation of labelled amino acids into the acid-precipitable protein of tissue homogenates will continue for some time after the destruction of the cell (Winnick, Friedberg & Greenberg, 1948). In liver homogenates, amino acids are incorporated particularly rapidly into the protein of the microsome fraction (Siekevitz, 1952). In this respect the homogenate resembles the intact liver since, in the normal animal given isotope, the initial incorporation is mainly in the microsome fraction (e.g. Hultin, 1950). Zamecnik & Keller (1954) were able to obtain an active cell-free preparation from liver which consisted of only the microsome and soluble cell-sap fractions. In this preparation incorporation of [^{14}C]amino acids into microsome protein would proceed under anaerobic conditions, provided that an adenosine triphosphate-generating system was present. The amino acids incorporated into microsome protein *in vitro* are undoubtedly linked to protein by peptide linkage since labelled peptides can be isolated by partial hydrolysis of microsome protein (Zamecnik, Keller, Littlefield, Hoagland & Loftfield, 1956). It has not, however, been clearly established whether the incorporation of amino acids into protein in such cell-free systems involves the same mechanism as occurs in the intact cell.

The present paper provides some information concerning the difference between the incorporation process in the intact liver cell and in a cell-free system. The technique employed in this study was that of fractionation of the microsome material by successive extraction with solutions of varied ionic strength and pH. By this method a number of subfractions of distinct composition are obtained (Simkin, 1955; Simkin & Work, 1957). We have previously reported the application of this method to a study of the incorporation of [^{14}C]amino acids into the microsome fraction of the liver of the intact guinea pig, and it was established that amino acids were taken up into the proteins of the various subfractions at quite different rates (Simkin & Work, 1957). We have now applied this method to microsome material isolated after incubation with [^{14}C]-amino acids in a cell-free system of guinea-pig liver similar to the type described by Zamecnik & Keller (1954). A preliminary report of this work has been made (Simkin, 1957).

EXPERIMENTAL

Animals. Young guinea pigs from the Institute stock were used (400–500 g., about 4 months old). The animals were starved overnight before use.

Materials. All reagents, except K_2HPO_4 and 3-phosphoglycerate, were of analytical reagent grade. 3-Phosphoglycerate was purchased as the barium salt from C. F. Boehringer und Soehne, Mannheim. A mixture of uniformly ^{14}C-labelled L-amino acids was prepared by acid hydrolysis of [^{14}C]chlorella protein (Radiochemical Centre, Amersham, Bucks). The hydrolysate, after removal of HCl, was adjusted with NaOH to pH 7 before use. All solutions were prepared with deionized water.

Preparation and incubation of the cell-free system. The animals were killed by a blow on the head, and their livers removed and minced as described by Simkin & Work (1957). Liver mince was homogenized with three times its weight of 0·25 M-sucrose for 25 sec., with similar conditions of homogenization to those reported previously. The larger particles were removed from the homogenate by centrifuging twice as before; the resulting preparation is referred to as the microsome–cell-sap fraction (or preparation).

The microsome–cell-sap fraction (10 ml.) was added to 4·4 ml. of a 0·18 M-sucrose solution which contained KCl, 0·0655 M; $KHCO_3$, 0·082 M; K_2HPO_4, 0·045 M; KH_2PO_4, 0·00425 M; $MgCl_2$, 0·0082 M; potassium 3-phosphoglycerate 0·033 M; [^{14}C]chlorella protein hydrolysate, 0·00059 M, 2·71 μc/ml. The reaction mixtures were incubated at 37° with shaking in a vigorous stream of $N_2 + CO_2$ (95:5, v/v). After incubation, 1·5 vol. of an ice-cold 0·25 M-sucrose solution containing unlabelled amino acids (0·002 M with respect to casein hydrolysate N) were added and the mixture cooled in ice. In the experiment in which the specific activity of the acid-soluble fraction of the cell sap was investigated, the sucrose solution added at the end of incubation did not contain unlabelled amino acids.

Preparation of the microsome subfractions and cell sap. The microsome fraction was sedimented without delay by centrifuging at 30000 rev./min. ($g_{av.} = 78000$) for 60 min. in Rotor 30 of a Spinco Model L ultracentrifuge. The supernatant was removed as previously described and taken as the cell-sap fraction. The microsome pellets were washed with 0·25 M-sucrose as before and stored overnight at 2° (when

non-incubated material was fractionated, this was stored if necessary at $-10°$ for several days before fractionation).

Fractionation procedure. When microsome material was fractionated, the material obtained from four separate incubation flasks set up as described above was pooled together for extraction. The pooled material representing the microsome material from about 15 g. of liver was washed twice with 38% (v/v) glycerol as reported previously, but the volume chosen for each washing was about 38 ml. and the centrifugal force employed was 30 000 rev./min. ($g_{av.} = 78\,000$) for 30 min. in Rotor 30 of the Spinco Model L ultracentrifuge.

The fractionation procedure employed was essentially that of Simkin & Work (1957), but certain changes were made. The volume of solution used for the first extraction with either 0·14 M-NaCl or M-NaCl, and extraction with buffer (pH 9) or 0·01 N-NaOH, was about 12 ml. This corresponded to the volume of extractant/mg. of original N previously employed, but in the second extraction with 0·14 M-NaCl or M-NaCl 24 ml. of extractant was used (about two-thirds of the volume used previously). At each stage, the insoluble material was separated by centrifuging for 10 min. at 40 000 rev./min. ($g_{av.} = 105\,000$) in Rotor 40 of the Spinco Model L ultracentrifuge, except at the stage of separation into fractions *D* and *E*, where the time of centrifuging at this speed was reduced to 5 min.

In several experiments involving longer periods of incubation, the non-incubated microsome material was not fractionated, the specific activity of only the whole microsome protein being determined. In these cases, the washing procedure with 38% glycerol was omitted, the microsomes being suspended in iced water before the precipitation of protein.

Isolation of protein for radioactivity measurement and determination of radioactivity. Protein was precipitated with trichloroacetic acid (TCA), reprecipitated, purified and counted as described by Simkin & Work (1957). Under the conditions used 10^{-3} μc/mg. of acrylate resin gives 1000 counts/min. In Table 1 of Simkin & Work (1957) the standard was mistakenly referred to as carbon.

Determination of specific activity of the TCA-soluble fraction of the cell sap. To a sample of the cell-sap fraction at 0°, an equal volume of ice-cold 10% (w/v) TCA was added. The precipitate of protein was centrifuged down in the cold, and the supernatant removed and taken as the TCA-soluble fraction. The TCA-soluble fraction was diluted tenfold with water before determination of specific activity. The radioactivity of the diluted material was determined by evaporating a sample (0·1–0·2 ml.) on a 1 cm.^{2} polythene planchet containing a drop of very dilute detergent (to aid spreading of material over the surface of the planchet). The samples were then counted as described by Simkin & Work (1957). The 'amino N' content of a sample (4·0 ml.) of the diluted material was determined as described below.

Amino nitrogen. This was determined colorimetrically by the β-naphthoquinonesulphonate method of Frame, Russell & Wilhelmi (1943), as modified by Russell (1944). The reagents used were proportionately reduced to give a final total volume of 10 ml. A solution containing equimolar quantities of glycine and glutamic acid was used for determining a standard curve, as suggested by Frame *et al.* (1943). Reagent-blank values were determined by using solutions containing sucrose and TCA at concentrations equivalent to those present in the material assayed. Control experiments showed that such solutions occasionally caused a slight reduction (10% or less) in the colour values given by the standard solution containing glycine and glutamic acid. Duplicate determinations on assay material were in excellent agreement.

Protein and ribonucleic acid. These were estimated by methods similar to those reported by Simkin & Work (1957). The method of separation used was that described for estimation of ribonucleic acid and acid-soluble nucleotide; the protein remaining after extraction with M-$KClO_4$ at room temperature was dissolved in N-NaOH and estimated.

Ultraviolet-absorption curves. Ultraviolet-absorption curves were determined with a Unicam SP. 500 spectrophotometer.

RESULTS

Incorporation of [^{14}C]amino acids into microsome proteins in a cell-free system

The time course of incorporation of a mixture of [^{14}C]amino acids ([^{14}C]chlorella protein hydrolysate) into microsome protein when a microsome–cell-sap preparation of guinea-pig liver was incubated under the conditions described is shown in Fig. 1. Incorporation takes place in an approximately linear manner for about 20 min. Other data indicate that there is no change in specific activity of microsome protein between 20 and 40 min. Similar curves have been reported by Zamecnik & Keller (1954).

Fig. 2 shows the time course of incorporation of [^{14}C]amino acids into the proteins of microsome

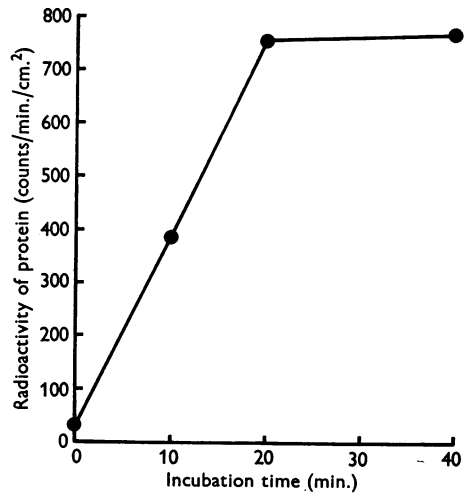

Fig. 1. Incorporation of [^{14}C]amino acids into microsome protein after incubation of a cell-free system prepared from guinea-pig liver. The incubation mixture contained: microsome + cell-sap fractions; KCl (0·020 M); $KHCO_3$ (0·025 M); potassium phosphate buffer, pH 7·8 (0·015 M); $MgCl_2$ (0·0025 M); potassium 3-phosphoglycerate (0·010 M); [^{14}C]chlorella protein hydrolysate (0·00018 M, 0·83 μc/ml.) For other details, see text.

subfractions in a cell-free system. The microsome material was fractionated by the procedure of Simkin & Work (1957) after incubation of the microsome–cell-sap preparations for different times. The preparations were not incubated for longer than 20 min., because linear incorporation continues only for about that time in this system (Fig. 1). A different microsome–cell-sap preparation was used for each time point studied; the 20 min. results are the mean values for two separate experiments (the values were in close agreement). In all experiments, a value for the incorporation of amino acids into the non-incubated microsome material was also obtained. In three experiments (with incubation periods of 10, 15 and 20 min.) the non-incubated microsome material was not fractionated; the values used for correcting for zero time uptake into the microsome subfractions were derived from the mean distribution pattern obtained from three other preparations (the mean zero-time incorporation values for the proteins of the subfractions were as follows, the whole microsome protein value being taken as 1·00: *A*, 0·22; *B*, 0·52; *C*, 0·45; *D*, 0·70; *E*, 1·29). Any error resulting from the use of the zero-time mean values will be small because the specific activity of the non-incubated microsome material was only about 10% of that of the incubated microsomes.

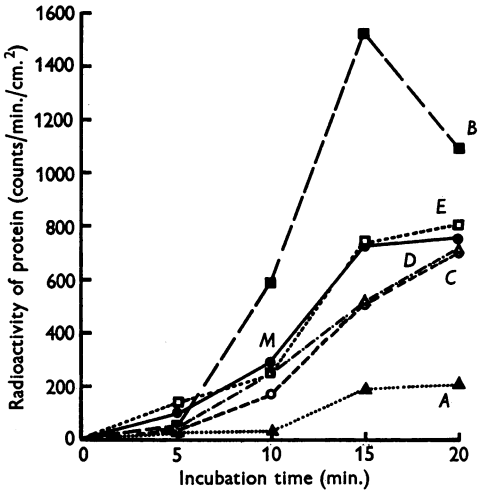

Fig. 2. Incorporation of [^{14}C]amino acids into the protein of microsome subfractions after incubation of a cell-free system from guinea-pig liver. The cell-free system was prepared and incubated as described in the text; after incubation, the microsome material was isolated and fractionated. The values have been corrected for zero-time uptake (see text). The specific activities of the proteins are represented by: whole microsome *M*, ●—●; fraction *A*, ▲···▲; fraction *B*, ■——■; fraction *C*, ○– – –○; fraction *D*, △—·—△; fraction *E*, □- - -□.

Fig. 2 shows that the protein of microsome subfraction *A* (extracted with 0·14 M-NaCl) had, throughout the whole period studied, a much lower activity than that of the whole microsome protein. The protein of fraction *B*, a ribonucleoprotein subfraction extracted by M-NaCl, had an activity only about one-half that of the whole microsome protein at both zero time and at 5 min.; but there then followed a rapid increase in specific activity, so that at 10 and 15 min. the protein had an activity about twice that of the whole microsome protein, and represented about 25% of the total radioactivity of the whole microsome protein (cf. about 6% at 5 min.). The radioactivity had declined somewhat from its maximum level after 20 min. incubation. In contrast, the protein of fraction *C*, another ribonucleoprotein subfraction isolated by extraction with 0·1 M-bicarbonate–carbonate buffer (pH 9), showed a gradual increase in activity during incubation, the value gradually approaching but not exceeding the whole microsome-protein value. The radioactivity of this fraction represented some 5% of the total after 20 min. incubation. The lipid-containing fractions *D* and *E*, separated by their differing solubility in 0·01 N-NaOH, showed a somewhat different pattern of labelling. The protein of fraction *E* had an activity higher than that of the whole microsome protein at zero time and after 5 min. incubation, whereas the activity of fraction *D* was somewhat lower. Further incubation beyond 5 min. resulted in a gradual increase in the activity of both subfractions, approximately in proportion to the increase in the whole microsome-protein activity, fraction *E* being found to have an activity approximately equal to that of the whole and *D* somewhat lower. At both 0 and 5 min. the proteins of fractions *D* and *E* together accounted for about 90% of the radioactivity of the whole microsome protein, but after 10 and 15 min. incubation this proportion had fallen to 70%.

Significance of incorporation of [^{14}C]*amino acids into microsome proteins in a cell-free system*

The results quoted above, in particular those for the pattern of incorporation of amino acids into the proteins of microsome subfractions *B* and *C*, are quite different from those reported earlier (Simkin & Work, 1957) for the labelling of these subfractions *in vivo* (Fig. 3). It was therefore necessary to establish that the subjection of microsome material to the conditions of incubation did not result in artifacts, such as a redistribution or loss of radioactivity. The following control experiment was therefore carried out. [^{14}C]Chlorella-protein hydrolysate (15 μc/kg. body weight, 3·1 μmoles of N/kg. body weight) was injected into two guinea pigs (mean weight, 510 g.), which were killed 30 min. later, at which time the radioactivity of the protein of

microsome subfraction *C* would be expected to be at or near its maximal level (Simkin & Work, 1957). The microsome–cell-sap fraction was isolated from the pooled livers in the usual way and incubated for 10 min. under the conditions previously described, except that no [^{14}C]amino acids were added to the incubation mixture. The values for incorporation into the proteins of the microsome subfractions subsequently isolated in this experiment are shown in Table 1, together with a comparison of results previously obtained for two other guinea pigs killed 30 min. after injection of labelled amino acids. It will be seen that there is little change in the specific activity of the whole microsome or cell-sap proteins as a result of incubation. The radioactivity of the TCA-soluble material of the cell sap present during the incubation of this sytem was about 0·5 % of that normally present in the complete system *in vitro*. There was in general little change in the distribution of radioactivity between the proteins of the microsome subfractions when compared with the non-incubated values. Such differences as were found are probably due to variations between individual animals; the animals used in the incubation experiment were about half of the weight of those used previously. It would be expected that the smaller animals would have the higher rate of metabolism, and the differences found between the two sets of values are consistent with this supposition. It would thus appear probable that incorporation data obtained in cell-free systems under the conditions described and involving periods of incubation similar to those employed would give a substantially correct measure of the actual pattern of labelling.

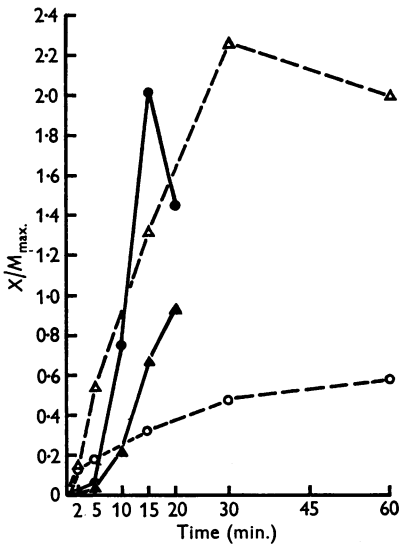

Fig. 3. Comparison of the incorporation of [^{14}C]amino acids into the proteins of microsome subfractions *B* and *C* in a cell-free system prepared from guinea-pig liver with that occurring in the liver of the intact guinea pig. Radioactivity is expressed as the ratio of the specific activity of the subfraction protein (*X*) to that of the highest complete microsome-protein value found in the time considered ($M_{max.}$) (20 min. value for the cell-free system and 60 min. value for the intact animal). Fraction *B in vitro*, ●—●; fraction *C in vitro*, ▲—▲; fraction *B in vivo*, ○— —○; fraction *C in vivo*, △— —△.

Some data have also been obtained relating to the composition of the microsome material isolated from the cell-free systems after varying periods of incubation. The ultraviolet-absorption spectra of the microsome subfractions were determined in all experiments, and the protein and ribonucleic acid (RNA) content of whole microsome material and also of the subfractions was determined in several. It was found that, while there was little significant change in the total quantity of microsome protein after periods of incubation of up to 40 min. duration, there was some loss of microsome RNA, amounting after 30 min. incubation to about 35 % of the RNA present in the zero-time control. As the composition of the subfractions isolated from microsome material incubated for only short periods was comparable to that of the corresponding zero-time control materials, it is probable that the loss of RNA is distributed throughout the various subfractions, as this would result in only a relatively small decrease in the RNA content of each subfraction. Some evidence was obtained suggesting that the RNA of

Table 1. *Distribution of radioactivity among proteins of liver-microsome subfractions obtained from guinea pigs killed* 30 *min. after intravenous injection of* [^{14}C]*amino acids*

In (I), the microsome–cell-sap fraction was incubated, without further addition of [^{14}C]amino acids, for 10 min. under the conditions described in the text. The values in parentheses are those of zero-time controls. (II) represents values found previously (Simkin & Work, 1957) for subfractions labelled *in vivo*: no incubation step was included. The results are expressed as the ratio of the specific activity of the protein isolated to that of the corresponding whole microsome protein. *M* represents the protein of the whole microsome fraction and *S* that of the cell sap; *A*, *B*, *C*, *D* and *E* represent the proteins of the various microsome subfractions.

	M	*S*	*A*	*B*	*C*	*D*	*E*
(I) Incubated	1·00 (0·94)*	0·24 (0·24)	1·54	0·56	1·92	0·88	0·73
(II) Non-incubated	1·00	0·24	1·08	0·50	2·36	0·88	0·70

* Washed with aqueous 38 % (v/v) glycerol.

microsome subfraction *C* is more readily lost than that of subfraction *B*. The ratio of the extinction at 260 mμ to that at 280 mμ of fraction *B* was found to be similar in microsome material isolated after periods of incubation of 20 min. or less; the mean of 11 values was 1·86 ± S.D. 0·15. In fraction *C*, however, while there was little difference in this ratio after incubation for periods of up to 10 or 15 min. (mean of eight values for periods of 10 min. or less: 1·82 ± S.D. 0·12), after 20 min. incubation, lower values (1·39, 1·55) were found, suggesting a lower RNA/protein ratio. The ultraviolet-absorption spectra of subfractions obtained from incubated microsome materials were in other respects similar to those from zero-time materials, thus indicating that the loss of RNA found is probably the major change occurring as a result of incubation, and also that the microsome material is not altered in such a way as to interfere with the fractionation procedure.

In the animals used for the present study, the amount of fraction *C* obtained was relatively somewhat smaller than was found in the animals used for the *in vivo* studies previously reported (Simkin & Work, 1957), fraction *C* representing only about 5% of the whole microsome protein, as compared with some 10–15% previously found. The RNA/protein ratio was, however, of the same order as that previously reported. The microsome fraction in the experiment in which material labelled *in vivo* was incubated (Table 1) had a relatively low content of fraction *C*; the incorporation data obtained do, however, indicate that the pattern of incorporation into the proteins of the microsome subfractions is essentially similar to that reported previously. A relatively low proportion of fraction *C* protein has also been found in other recently fractionated microsome material which had not been subjected to incubation (Bhargava, Simkin & Work, 1958).

Incorporation of [^{14}C]*amino acids into cell-sap proteins in a cell-free system*

It will be seen from Fig. 4 that there is a significant increase in the specific activity of the soluble proteins of the cell sap as a result of incubation of the cell-free system used. There appears to be little increase in specific activity during the first 10 min. of incubation, but a rapid increase in activity then occurs, the extent of the increase being about 10% of the increase in the activity of the whole microsome protein in the same preparation. There is possibly some increase in activity of cell-sap protein after incorporation into whole microsome protein has ceased.

Specific activity of the TCA-soluble fraction of the cell sap

In view of the importance of a knowledge of the specific activity of precursor material for the interpretation of incorporation data, some information on this important parameter was obtained by determining the specific activity of the TCA-soluble material of cell sap isolated after different periods of incubation of the complete incorporation system. The data have been expressed in terms of counts/min./μm-mole of 'amino N'. The colorimetric method of Frame *et al.* (1943) used for the determination of amino nitrogen does not have such a high degree of specificity for amino acids as does

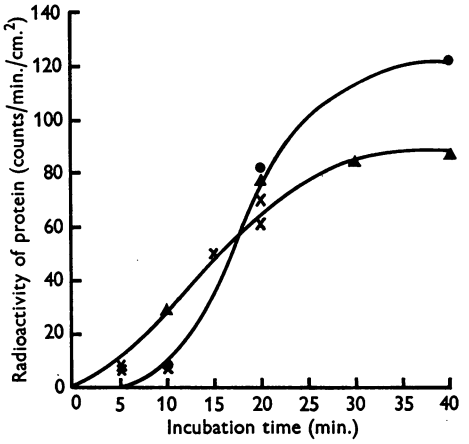

Fig. 4. Increase in specific activity of the protein of the cell-sap fraction during incubation of a microsome–cell-sap preparation from guinea-pig liver with [^{14}C]amino acids. The values have been corrected for zero-time activity. The data shown include two time curves obtained with single microsome–cell sap preparations (●, preparation 1; ▲, preparation 2), as well as values obtained in separate experiments in which each preparation was used for one time point only (×). The cell-sap fractions from preparation 1 were isolated from the same cell-free systems as the microsome material used to obtain the data shown in Fig. 1.

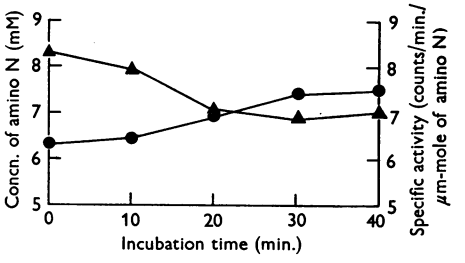

Fig. 5. Changes in the trichloroacetic acid (TCA)-soluble material of the cell sap during incubation of a microsome–cell-sap fraction, prepared from guinea-pig liver as described in the text, with [^{14}C]amino acids. ●, Concentration of the TCA-soluble amino N of the cell sap; ▲, specific activity of the TCA-soluble fraction in terms of amino N.

the ninhydrin–CO_2 method of Van Slyke, Dillon, MacFadyen & Hamilton (1941), but it was considered that the approximate values obtained by the colorimetric method would indicate whether large changes in specific activity were taking place during the period of incubation. It will be seen from Fig. 5 that there is a slight increase in the TCA-soluble amino N level of the cell sap during incubation of the system; as there is little change in the total TCA-soluble radioactivity, this results in a corresponding diminution of specific activity.

Amino acid composition of the proteins of microsome subfractions B and C

Because of the marked differences in the metabolic behaviour of the proteins of microsome subfractions *B* and *C*, and because of their possible importance in the mechanism of amino acid incorporation, an analysis was made of their amino acid composition (Table 2). The analyses were performed by Dr S. Jacobs, using a modified Moore & Stein method (Dr S. Jacobs, unpublished work). It will be seen that there are some differences in the content of a number of amino acids, thus suggesting that the subfractions represent mixtures of different proteins. The relative quantities of the amino acids present are similar to those found in many saline-soluble mammalian proteins (Müting & Wortmann, 1954). The proteins do not appear to be characterized by a high content of basic amino acids (cf. Allfrey, Daly & Mirsky, 1953).

Table 2. *Amino acid composition of the protein of microsome subfractions B and C*

The proteins analysed were the pooled materials isolated for radioactivity determination (Simkin & Work, 1957) in four or five separate experiments. The microsome fractions from which the proteins were isolated had not been subjected to incubation. The proteins were hydrolysed with 6N-HCl, and the amino acids of the hydrolysates separated on columns of Zeo-Karb 225, WR. 1·55 (see text). The results are given in terms of grams of amino acid/100 g. of total amino acid recovered (the tryptophan and cystine contents of the proteins were not determined).

Amino acid	Fraction *B*	Fraction *C*
Aspartic acid	9·5	11·7
Threonine	5·2	3·9
Serine	3·9	5·2
Glutamic acid	12·0	14·9
Proline	7·5	5·7
Glycine	4·7	4·6
Alanine	5·3	5·6
Valine	5·8	6·1
Methionine	2·0	1·8
Isoleucine	4·2	3·9
Leucine	10·2	8·3
Tyrosine	4·0	4·6
Phenylalanine	5·6	7·1
Lysine	9·3	8·1
Histidine	2·5	3·2
Arginine	8·3	5·3

DISCUSSION

The incorporation of labelled amino acids into protein has been used extensively in recent years as a criterion of protein synthesis. While data obtained with the intact animal are unlikely to be invalidated because of artifacts, the results are difficult to interpret because of the complexity of the experimental system, and it thus seemed desirable that protein synthesis should be studied in simpler subcellular systems. When information is obtained from cell-free systems, it is then desirable to compare this if possible with similar information from the intact animal in order to ascertain whether the mechanism of protein synthesis revealed in the subcellular system is in fact similar to that occurring in the intact cell. This poses the problem of how best an experiment can be devised in which data can be obtained relating to protein synthesis in a cell-free system, and in addition enabling such a comparison to be made. Probably the most desirable way in which to carry out such an investigation is to study the synthesis of a specific protein under both sets of conditions. It has, however, in general proved difficult to demonstrate synthesis of specific proteins as measured by either net synthesis or incorporation of labelled amino acids in cell-free preparations (cf. Askonas, Simkin & Work, 1957), although in a few instances some increase in enzymic activity in subcellular systems has been reported, for example, by Gale & Folkes (1955), using disrupted staphylococci, and by Ullmann & Straub (1955), using pancreas. It has not yet been clearly established whether these increases represent protein synthesis. The criterion of protein synthesis in cell-free systems has thus of necessity been the less satisfactory one of incorporation of labelled amino acids into acid-precipitable protein. The present investigation has been designed as an attempt to perform a comparative study of the type outlined above.

The subject of study has been the microsome fraction: this fraction is presumably of importance in the process of protein synthesis in the intact cell, because under these conditions there is a high rate of incorporation of amino acids into microsome protein. Study of this subcellular fraction *in vitro*, where a high turnover rate of protein is also found, might well provide further information on the mechanism of protein synthesis. A comparison has been made under the two sets of conditions between a series of apparently distinct microsome subfractions which are known to have quite varied rates of incorporation in the whole animal (Simkin & Work, 1957).

The results obtained in the present investigation show that in cell-free preparations there is a particularly rapid uptake of labelled amino acids into protein associated with RNA. In this respect we agree

with other workers who have used similar systems from rat liver (Littlefield, Keller, Gross & Zamecnik, 1955; Sachs & Waelsch, 1956). When, however, the incorporation data for the microsome subfractions are compared with those obtained for similar material labelled *in vivo*, it is seen that there are marked differences in the pattern of incorporation into the proteins of the various subfractions. In particular, the difference in behaviour of the ribonucleoprotein fractions *B* and *C* is striking (Fig. 3). In the cell-free system, the protein of fraction *B*, after an initial lag period, shows a very rapid increase in specific activity, whereas that of fraction *C* shows only a gradual increase in activity. In the intact guinea pig, on the other hand, the protein of fraction *C* increased rapidly in activity, whereas fraction *B* had a lower activity than the whole microsome protein during the entire period studied (up to 3 hr. after administration of labelled amino acid). The behaviour of the protein of fraction *A* under the two conditions is also quite different. The proteins of the lipid-containing fractions *D* and *E* do not show marked differences in the pattern of labelling in the two systems. In the cell-free system the protein of fraction *E* has a higher activity than the whole microsome protein during the early stages of incorporation, but this high activity is possibly due to non-specific labelling, perhaps resulting from the technical difficulties encountered in the isolation of protein from such lipoproteins. Thus, while incorporation into protein in a cell-free system resembles that in the corresponding intact cell in that the most rapid uptake of amino acids takes place into protein associated with RNA, the pattern of labelling obtained in cell-free preparations is quite different from that observed in the whole animal. The reasons for these differences are not clear; there are, however, several important differences between the two systems which may play a significant part in determining the altered pattern of incorporation in the cell-free system. The alteration in composition of the microsome fraction which takes place upon incubation may affect the amino acid incorporating system. As mentioned above, evidence has been obtained indicating that there is some loss of microsomal RNA upon incubation, perhaps involving in particular that associated with subfraction *C*. Since it is known that ribonuclease-treated microsomes show a low rate of amino acid incorporation (Allfrey *et al.* 1953; Zamecnik & Keller, 1954), the loss of RNA upon incubation may play a part in determining the pattern of incorporation obtained, and could also explain why incorporation ceases after about 20 min. incubation (cf. Canellakis, 1957). Another important difference between the conditions obtaining *in vivo* and in the cell-free system lies in the level of specific activity of the free amino acid pool during the incorporation process. In the intact animal, the specific activity of the free amino acids of the liver falls rapidly from an initial very high value found almost immediately after intravenous administration of tracer-doses of [^{14}C]amino acid (e.g. Humphrey & Sulitzeanu, 1958), whereas in the cell-free system the specific activity of the free amino acids remains to a first approximation constant (Fig. 5). Thus the cessation of incorporation in the cell-free system, found to occur after 20 min. incubation, is not the result of a fall in the specific activity of the amino acids of the medium.

In the cell-free system studied, after an initial lag period there appears to be some increase in the specific activity of the soluble protein of the cell sap. The extent of the increase found is somewhat larger than that reported in a similar system by Zamecnik & Keller (1954). The increase in the activity of the protein of the cell-sap fraction may represent genuine labelling of the soluble protein present; it will be of interest to ascertain whether specific proteins are labelled in this system. The relatively small increase in the activity of the soluble protein may explain the difficulty in demonstrating synthesis of specific proteins in subcellular systems (see above). The increase in activity of the soluble protein fraction could occur, however, merely as a result of a non-specific loss of labelled microsome protein. Since there is almost four times as much soluble protein as microsome protein in the system used, it may be calculated that the radioactivity of the cell-sap protein after 20 min. incubation is about 40 % of that of the total microsome protein at this time. As no appreciable loss of microsome protein could be detected, a non-specific increase in the activity of the cell-sap protein could be accounted for only by the loss of a small quantity of microsome protein of very high specific activity.

SUMMARY

1. A study has been made of the incorporation of [^{14}C]amino acids into microsome proteins in a cell-free system prepared from guinea-pig liver. The cell-free system, containing microsome and cell-sap fractions, was incubated for varying periods of time with a mixture of [^{14}C]amino acids; after incubation, the microsome material was isolated and fractionated by the extraction procedure of Simkin & Work (1957).

2. In this cell-free system, [^{14}C]amino acids are incorporated into the proteins of the microsome subfractions at quite different rates. The protein of a subfraction containing ribonucleic acid (RNA) (fraction *B*) attained a specific activity more than twice that of the whole microsome protein, whereas the protein of another RNA-containing subfraction (fraction *C*) showed a much slower increase in activity.

3. The data obtained have been compared with the results previously reported from the application of the same fractionation technique to guinea-pig-liver microsome material labelled *in vivo*. The pattern of incorporation into the subfractions found in the cell-free system is quite different from that found in the intact liver cell: in particular, the behaviour of the proteins associated with RNA is markedly different.

4. Changes which occur in the microsome material as a consequence of incubation have been invcstigated: an appreciable loss of RNA was found to occur. The significance of the incorporation of [^{14}C]amino acids into microsome protein in this cell-free system has been discussed.

5. A brief study was also made of the changes in the specific activity of both the soluble protein and of the trichloroacetic acid-soluble material of the cell-sap fraction after incubation of this cell-free system with [^{14}C]amino acids.

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REFERENCES

Allfrey, V. G., Daly, M. M. & Mirsky, A. E. (1953). *J. gen. Physiol.* **37**, 157.
Askonas, B. A., Simkin, J. L. & Work, T. S. (1957). *Symp. biochem. Soc.* no. 14, p. 32.
Bhargava, P. M., Simkin, J. L. & Work, T. S. (1958). *Biochem. J.* (in the Press).
Canellakis, E. S. (1957). *Biochim. biophys. Acta*, **23**, 217.
Frame, E. G., Russell, J. A. & Wilhelmi, A. E. (1943). *J. biol. Chem.* **149**, 255.
Gale, E. F. & Folkes, J. P. (1955). *Biochem. J.* **59**, 675.
Hultin, T. (1950). *Exp. Cell Res.* **1**, 376.
Humphrey, J. H. & Sulitzeanu, D. (1958). *Biochem. J.* (in the Press).
Littlefield, J. W., Keller, E. B., Gross, J. & Zamecnik, P. C. (1955). *J. biol. Chem.* **217**, 111.
Müting, D. & Wortmann, V. (1954). *Biochem. Z.* **325**, 448.
Russell, J. A. (1944). *J. biol. Chem.* **156**, 467.
Sachs, H. & Waelsch, H. (1956). *Biochim. biophys. Acta*, **21**, 188.
Siekevitz, P. (1952). *J. biol. Chem.* **195**, 549.
Simkin, J. L. (1955). *Commun. 3rd Int. Congr. Biochem., Brussels*, p. 74.
Simkin, J. L. (1957). *Biochem. J.* **66**, 6P.
Simkin, J. L. & Work, T. S. (1957). *Biochem. J.* **65**, 307.
Ullmann, A. & Straub, F. B. (1955). *Acta physiol. hung.* **8**, 279.
Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 627.
Winnick, T., Friedberg, F. & Greenberg, D. M. (1948). *J. biol. Chem.* **175**, 117.
Zamecnik, P. C. & Keller, E. B. (1954). *J. biol. Chem.* **209**, 337.
Zamecnik, P. C., Keller, E. B., Littlefield, J. W., Hoagland, M. B. & Loftfield, R. B. (1956). *J. cell. comp. Physiol.* **47**, suppl. 1, 81.

Polyphosphates Excreted by Wax-Moth Larvae (*Galleria mellonella* L. and *Achroia grisella* Fabr.)

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During their studies on the biochemistry of *Galleria mellonella*, the greater wax-moth, Niemierko (1950) and Niemierko & Niemierko (1950*a*, *b*) observed that these organisms excreted large amounts of an acid-labile phosphate which was not present in their food. They identified this material as inorganic polyphosphate, and showed that 85% of the total orthophosphate consumed was excreted in this form. Polyphosphates are also formed by *Achroia grisella*, the lesser wax-moth, but not by a number of other insects, suggesting that the formation of these compounds is connected with the ability to digest and assimilate wax.

Although the polyphosphates are widespread in bacteria, moulds and algae (Schmidt, 1951), Niemierko's work is, so far, the only convincing identification of these compounds in material derived from higher animals. Niemierko & Niemierko (1950*a*, *b*) present some evidence that they were not produced by intestinal bacteria. It has been claimed that small amounts of polyphosphates occur in the fat bodies of other insects (Heller, Karpiak & Zubikowa, 1950), but details of the identification were not given. As large amounts of the excreta of both wax-moths were available, it seemed worth while to confirm the results of Niemierko & Niemierko and to attempt to identify the polyphosphates present by ion-exchange chromatography.

EXPERIMENTAL

The excreta of both moths were provided by Dr L. Bailey. The moths were cultured at either 35° (*G. mellonella*) or room temperature (*A. grisella*) in large glass jars containing